

AMENDMENTS TO THE SPECIFICATION

Please amend the title as follows, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

MODULATORS OF P-SELECTIN GLYCOPROTEIN LIGAND-1 APOPTOSIS-INDUCING ANTI-PSGL-1 ANTIBODY COMPOSITIONS FOR THERAPEUTIC USE

Please replace the paragraph bridging pages 11-12 with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

PSGL-1 is a cell surface adhesion molecule that is expressed on neutrophils, T and B-lymphocytes, NK cells, monocytes, dendritic cells, and primitive human CD34 hematopoietic progenitor cells. Through its ability to interact with selectins, PSGL-1 mediates the rolling of leukocytes on the endothelium and the extravasation of leukocytes into inflamed tissues. PSGL-1-mediated binding of T cells to E- and P-selectin, or migration, is differentially regulated. For instance, the appearance of CLA (cutaneous lymphocyte antigen) epitope is thought to be induced on T cells undergoing naive to memory transition. Only activated helper 1 but not helper 2 T cells express functional PSGL-1 and ~~it~~ are capable of migration into the inflamed area of the skin.

Please replace the paragraph on page 15, lines 3-7, with the following paragraph, where text to be added is indicated by underlining:

The antibodies can be used, for example, as part of a therapeutic regimen (e.g., to reduce or eliminate an undesirable immune response, such as a T cell mediated immune response, associated with conditions such as inflammatory diseases, autoimmune diseases, transplant rejection, allergic diseases, and T cell-derived cancers). Antibodies also can be used in a screening assay to measure the ability of a candidate compound to bind to PSGL-1.

Please replace the paragraph on page 19, lines 14-22, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

Such compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (Lam et al., Nature 354:82 [1991]; Houghten et al., Nature 354:84 [1991]), and combinatorial chemistry-derived molecular library made of D- and/or L configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate, directed phosphopeptide libraries; Songyang et al., Cell 72:767 [1993]), antibodies (including, but not limited to, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and ~~FAb Fab~~, ~~F(ab')₂~~ F(ab')₂ and ~~FAb Fab~~ expression library fragments, and epitope-binding fragments thereof), and small organic or inorganic molecules.

Please replace the paragraph bridging pages 22-23 with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

Examples of conditions that can be treated with the anti-PSGL-1 compounds described herein include, but are not limited to, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, and psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus ~~erythematosus~~ erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, type I diabetes, inflammatory bowel diseases, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue,

lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, interstitial lung fibrosis, graft-versus-host disease, cases of transplantation (including transplantation using allogeneic or xenogeneic tissues) such as bone marrow transplantation, liver transplantation, or the transplantation of any organ or tissue, allergies such as atopic allergy, AIDS, and T-cell neoplasms such as leukemias and/or lymphomas.

Please replace the paragraph on page 24, lines 5-14, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

A TAIP-specific monoclonal antibody was generated by applying the well known cell fusion methods of Kohler and Milstein ((1976) European Journal of Immunology 6:511-519) to produce a hybridoma secreting desired antibodies. Antibody-producing cells from a hamster injected with ~~Coneanevalin~~ Concanavalin A (Con A)-activated Balb/c spleen T cells were fused with a myeloma cell line to form an antibody secreting hybridoma. The two populations of cells were fused with polyethylene glycol, and the resulting antibody producing cells were cloned and propagated by standard tissue culture methods. One hybridoma generated according to these methods secreted a monoclonal antibody, designated TAB4, that was able to induce T cell apoptosis *in vitro* and deplete T cells *in vivo*. The protein recognized by TAB4 was designated T cell apoptosis inducing protein (TAIP).

Please replace the paragraph on page 25, lines 12-21, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

The spleen cells were adjusted to a final concentration of 3×10^6 /ml with RPMI medium and ~~Coneanevalin~~ Concanavalin A was added to a final concentration of 2 micrograms/ml to activate the T cells. The cell suspension was transferred to a 6-well culture plate (5 ml/well) or a 10-cm culture dish (10 ml/dish) and incubated at 37°C, 5% CO₂ for 48 hours before harvesting. The activated spleen cells, including activated T cells, were resuspended in 5 ml of HBSS and carefully overlaid on top of a 5 ml 55% cushion of Percoll solution in a centrifuge tube. Care

was taken not to disturb the separated layers. The cells were spun at 1,900 x g for 13 minutes at 25°C without the brake. The enriched T cells were collected from the interface of the two layers, washed twice with HBSS, and were ready for experiments.

Please replace the paragraph on page 26, lines 3-11, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

After an incubation period of 18-24 hours, the extent of apoptosis in each culture was determined using the 7-AAD apoptosis assay. The treated cells were transferred to FACS tubes (Falcon), washed twice with ice-cold FACS solution (1% fetal bovine serum, 0.05% sodium azide in PBS), pelleted at 200 x g at 4°C. The cells were resuspended in ice-cold FACS solution to a final concentration of $1-2 \times 10^7$ cells/ml. For staining, 0.1 ml of the resuspended cells were mixed with 7-AAD to a final concentration of 2 ug/ml and then incubated at 4°C in the dark for 20 minutes. Finally, the stained cells were washed twice with ice-cold FACS solution, resuspended in 0.5 ml of FACS solution and analyzed with BD™ LSR flow cytometer (~~Beckton Dickinson~~ Becton Dickinson).

Please replace the paragraph on page 27, lines 2-16, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

Cells were washed twice with ice-cold FACS solution (1% fetal bovine serum, 0.05% sodium azide in PBS) and spun at 200 x g at 4°C in a FACS tube (Falcon). The cells were resuspended in ice-cold FACS solution to a final concentration of 1×10^7 cells/ml and a 0.1 ml aliquot of the resuspended cells in a FACS tube (Falcon) was used for each assay. For surface staining, the TAB4 monoclonal antibody or a control hamster Ig at a final concentration of 2 ug/ml were added to the cells and the mixtures were incubated at 4°C for 30 minutes in the dark. The cells were washed once with ice-cold FACS and then stained with: (1) for spleen cells, cyochrome-conjugated anti-CD3 antibody (2 ug/ml), FITC-conjugated anti-hamster Ig, and PE-conjugated anti-CD8/CD4/CD19/CD11b/pan-NK/I-A/I-E/Mac-3 antibody (2 ug/ml) in 100 ul of ice-cold FACS solution; and (2) for thymus cells, FITC-conjugated anti-hamster Ig, PE-

conjugated anti-CD8, and cychrome-conjugated anti-CD4 antibodies (2 ug/ml) in 100 ul of ice-cold FACS solution. The reaction was performed at 4°C for 30 minutes in the dark. Finally, the stained cells were washed twice with ice-cold FACS solution, resuspended in 1 ml of FACS solution and analyzed with BD™ LSR flow cytometer (~~Beekton-Dickison~~ Becton Dickinson).

Please replace the paragraph bridging pages 29-30 with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

For FACS assays, the cells were fixed with 2% paraformaldehyde at 4°C for 20 minutes, washed twice, and resuspended in ice-cold FACS solution to a final concentration of 1×10^7 cells/ml. A 100 ul aliquot of the resuspended cells in a FACS tube (Falcon) was used for each assay. TAB4 or control hamster Ig at a final concentration of 2 ug/ml were added to the cells and the mixtures were incubated at 4°C for 30 minutes in the dark. The cells were washed once with ice-cold FACS and reacted with: (1) for spleen cells, cychrome-conjugated anti-CD3 antibody (2 ug/ml), FITC-conjugated anti-hamster Ig and PE-conjugated anti-CD8/CD4/CD19/CD11b/pan-NK1-A/I-E/Mac-3 antibody (2 ug/ml) in 100 ul of ice-cold FACS solution; and (2) for thymus cells, FITC-conjugated anti-hamster Ig, PE-conjugated anti-CD8, and cychrome-conjugated anti-CD4 antibodies (2 ug/ml) in 100 ul of ice-cold FACS solution. The reaction was performed at 4°C for 30 minutes in the dark. Finally, the stained cells were washed twice with ice-cold FACS solution, resuspended in 1,000 ul of FACS solution and analyzed with BD™ LSR flow cytometer (~~Beekton-Dickison~~ Becton Dickinson).

Please replace Table 2 (lines 12-15) on page 30 with the following, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

TABLE 2

Spleen				
x10 ⁶	No Treatment	Normal Hamster Ig	TA-B4 <u>TAB4</u> -treated	Depletion (%)
Total Splenocytes	123	93.3	105	14.6
CD3 ⁺ T cells	32.8	28.4	12.4	62.2
CD3 ⁺ CD19 ⁺	72.2	53.4	72.9	-0.8
CD3 ⁺ NK ⁺	3.6	2.4	1.80	50

Peripheral Blood Leukocytes				
	No Treatment	Normal Hamster Ig	TA-B4 <u>TAB4</u> -treated	Depletion (%)
CD3 ⁺ T cells	36.7%	36%	4.1%	88.8%

Thymus				
x10 ⁶	No Treatment	Normal Hamster Ig	TA-B4 <u>TAB4</u> -treated	Depletion (%)
Total Thymocytes	94	229	45	52.1
CD4 ⁺	9.3	28.4	10.9	-16.6
CD8 ⁺	5.2	7.7	3.6	30.3
CD4 ⁺ CD8 ⁺	73.8	182	26	64.7
CD4 ⁺ CD8 ⁻	5.6	10.5	4.5	19.3

(representative data from three experiments)

Please replace the paragraph bridging pages 31-32 with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

P-selectin glycoprotein ligand-1 (PSGL-1), also named CD162, is the main P-selectin ligand expressed on leukocytes, including T cells (Sako et al. (1993) Cell 75:1179; Vachino et al. (1995) J. Biol. Chem. 270:21966; Veldman et al. (1995) J. Biol. Chem. 270:16470). Biochemical characteristics of TAIP, such as its molecular weight and its tendency for dimerization suggested the possibility that ~~TAB4~~ TAIP may be analogous to PSGL-1. To investigate the relationship between these two antigens, the following were tested: 1) whether the

antigen precipitated by TAB4 can be recognized by a commercially-available anti-PSGL-1 anti-PSGL-1 antibody; and 2) whether an ~~anti-PSGL-1~~ anti-PSGL-1 antibody can deplete TAB4 TAIP from the cell lysate.

Please replace the paragraph on page 32, lines 7-20, with the following paragraph, where text to be added is indicated by underlining:

RL β 1 T cells were lysed at a density of 1.0×10^8 cells/ml in lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 160 mM NaCl, 1 mM CaCl₂) containing complete protease inhibitor cocktail for 1 hour, and insoluble material was pelleted at 10,000 x g for 10 minutes. These and all subsequent steps were performed at 4°C. The lysate corresponding to 5.0×10^7 cells was incubated with 20 μ l of protein G-Sepharose preloaded with 10 μ g of anti-PSGL-1 mAb (clone 2PH1, PharMingen, San Diego, Calif.), anti-TAIP mAb, TAB4, or IgG from normal hamster serum. After incubation for 4 hours at 4°C, the beads were washed five times with washing buffer (0.05% Triton X-100, 50 mM Tris-HCl, pH 8.5, 400 mM NaCl, 1 mM CaCl₂, 1 mg/ml ovalbumin), and twice with a similar washing buffer, containing 250 mM instead of 400 mM NaCl. Bound proteins were eluted with 40 μ l of 1xSDS sample buffer. Eluted proteins were separated by 6 % SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were immunoblotted with anti-PSGL-1 mAb, and revealed by peroxidase-conjugated goat anti-rat IgG (H+L) followed by chemiluminescence (Renaissance™, NEN).

Please replace the paragraph on page 33, lines 8-18, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

Human peripheral blood was taken from healthy adults, ~~heprinized~~ heparinized, and enriched for peripheral blood mononuclear cells (PBMC) based on differential density using Ficoll-Paque® ~~Plus~~ PLUS (Pharmacia Biotech). The PBMC were activated with 1% PHA (Life Technologies, GibcoBRL) for 48 hours and subsequently maintained in recombinant human IL-2 (5 ng/ml) through the assay period. To assess the apoptosis-inducing ability an anti-human PSGL-1 antibody, the activated cells were treated with: (1) 1 μ g/ml of the anti-PSGL-1 antibody

clone KPL-1 (BD PharMingen) plus cross-linker rabbit anti-mouse Ig (0.5 ug/ml) (Jackson ImmunoResearch Laboratories); (2) isotype control purified mouse Ig plus cross-linker rabbit anti-mouse Ig; or (3) cross-linker rabbit anti-mouse Ig alone. After six hours of treatment, the percentage of early apoptotic cells was determined by FACS, staining with anti-Annexin V (BD PharMingen) and PI (Sigma).

Please replace the paragraph on page 33, lines 19-23, with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

As shown in Fig. 8, signaling triggered by PSGL-1 using an anti-PSGL-1 antibody plus the crosslinker triggered significant level of apoptosis in PHA-activated human PBMC (mainly T cells). The percentage of apoptotic cells increased from 8.5% on days 3 to 24% on day 8 in anti-PSGL1 treated cultures. Neither ~~isotopic~~ isotype-matched control, nor the cross-linking antibodies alone, had any effect on these cells.

Please replace the paragraph bridging pages 33-34 with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

Non-obese diabetic (NOD) mice, a well-accepted autoimmune diabetes animal, were bred under standard conditions. Spontaneous diabetes developed in the NOD mice at the age of about 20 weeks. In the experimental group, the mice received three doses of anti-PSGL-1 antibody (TAB4) intraperitoneally at 300 µg per mouse at age of 14, 15 and 17 weeks. Two additional injections with the same dose were given at the ages of 24 and 26 weeks. The control group was given hamster Ig at the same dose. Mice were monitored for ~~glucose-uria~~ glucosuria by Medi-Test Glucose strips (Macherey-Nagel, Germany) twice every week after the age of 15 weeks. Non-fasting urine glucose levels over 300 mg/dl for two consecutive measurements were considered diabetic.

Please replace the paragraph on page 34, lines 19-28, with the following paragraph, where text to be added is indicated by underlining:

To assay the samples by FACS analysis, 2×10^5 cells per well from days 0, 2, 4 and 6 were incubated at 4°C for 30 minutes with 40 ul/well of mouse P-Selectin, E-Selectin, or L-Selectin fused to the Fc region of human IgG1 (R&D Systems, Minneapolis, Minn.) at concentrations ranging from 20 ug/ml with two-fold serial dilution to 0.156 ug/ml. Following the incubation, cells were washed with 1x FACScan® (Becton Dickinson) buffer (1 x PBS without calcium and magnesium ions from Biochrom AG, Berlin and 2% FBS). Samples were further incubated at 4°C for 30 minutes with 95 ul/well of anti-Thy1.2 and a secondary reagent (FITC-anti human IgG, which is specific to Fc fragment, purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) at 3.25 ug/ml, and then washed with 1x FACScan® (Becton Dickinson) buffer.

Please replace the paragraph bridging pages 34-35 with the following paragraph, where text to be added is indicated by underlining and text to be deleted is indicated by strikethrough:

The results of FACSCalibur™ (Becton Dickinson) analysis are shown in Fig. 10. At 20 ug/ml, binding of P-selectin to mouse activated T cells increased gradually, peaked on day 4, and declined slightly on day 6. Binding of E-selectin rose significantly from day 2 to day 4 and then remained peaked at day 6. Binding of L-selectin to mouse activated T cells was not apparent, and did not change through the activation period, i.e. from day 0 to day 6. The results observed with ~~L-Selectin~~ L-selectin could be due to the apparent low binding affinity of L-selectin to its ligand. Similar results were also obtained when lower concentrations of the three selectins were employed in the experiments.

Please replace the paragraph on page 35, lines 9-21, with the following paragraph, where text to be added is indicated by underlining:

A 96-well plate (NUNC™) was coated with 50 μ l of anti-human Fc Ig at 20 μ g/ml in 1 x PBS at 4°C overnight, blocked with 1% BSA at 37°C for 2 hours and incubated with 50 μ l of a selectin-human Fc fusion (from 0.063 to 5 μ g/ml) at room temperature for 2 hours. In all experimental steps, each well was thoroughly washed five times with 1 x PBS. Then 2×10^5 T cells activated previously with Con A for four days were added into each well and incubated at 37°C for 5 hours prior to centrifugation of the plate at 200 x g for 5 minutes at 4°C. The resulting pellet containing activated T cells was incubated with Annexin V-biotin conjugate at room temperature for 15 minutes and subsequently with an avidin conjugate (SA-beta-gal at 1:5000 dilution) for another 30 minutes at 37°C. In every binding reaction, each well was washed thrice with Annexin V binding buffer. The color development was achieved by incubating both 110 μ l of Z-buffer mixture (54 μ l of 2-mercaptoethanol in 20 ml of Z-buffer) and 30 μ l of ONPG (0.04 g/10 ml) at 4°C overnight. The readings of optical density at 420 nm were recorded.